TITLE

PHYTIC ACID BIOSYNTHETIC ENZYMES

~~This application claims the benefit of U.S. Provisional Application No. 60/082,960, filed April 24, 1998.~~

FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding phytic acid biosynthetic enzymes in plants and seeds.

BACKGROUND OF THE INVENTION

Myo-inositol 1,2,3,4,5,6-hexaphosphate, commonly known as phytic acid, is an abundant molecule in many plant seeds and vegetative tissue such as roots and tubers (Hartland and Oberlaeas (1986) *J. Assoc. Off. Anal. Chem. 69*:667-670). Phytic acid exists primarily as mixture of potassium, calcium, iron, zinc and magnesium phytate salts (Pernollet J. C. (1978) *Phytochemistry 17*:1473-1480).

In corn (*Zea mays L.*), 90% of the phytate is deposited in protein bodies localized in the germ whereas in legume crops 90% of the phytate is localized in the endosperm and cotyledons. Up to 80% of phytate is in the aluerone layer of wheat (*Triticum aestivum Lam.*) and rice (*Oryza sative L.*) (O'Dell B. L. et al. (1972) *J. Agric. Food Chem. 20*:718-721). The presence of phytate phosphorous in such food crops decreases the bioavailability of zinc by forming a very stable insoluble phytate zink complex, making the zinc unavailable in the intestinal mucosa of mammals (O'Dell, B. L., et al. (1972) *J. Agr. Food Chem. 20*:718-721). Although phytate phosphorous is readily available to ruminants, it is poorly available to monogastric animals. In addition to being only partially digestible, the presence of phytic acid in food crops leads to excretion of other limiting nutrients such as essential amino acids, calcium and zinc (Mroz, Z. et al. (1994) *J. Animal Sci. 72*:126-132; Fox et al., In Nutritional Toxicology Vol. 3, Academic Press, San Diego (1989) pp. 59-96).

Phytic acid is thought to arise in plants by two pathways. The first pathway uses free *myo*-inositol as the initial substrate, with subsequent phosphorylation by a phosphoinositol kinase. Contribution to the free *myo*-inositol pool is either by recycling from other pathways or by the dephosphorylation of *myo*-inositol-1-phosphate. The alternate pathway uses *myo*-inositol-1-phosphate as the initial substrate, with subsequent phosphorylations catalyzed by phosphoinositol kinase. The committed step for *myo*-inositol-1-phosphate production is the NAD^+-catalyzed oxidation of carbon 5 of the b-enantiomer of D-glucose-6-phosphate. This reaction is catalyzed by *myo*-inositol-1-phosphate synthase (Raboy, V. In Inositol Metabolism in Plants (1990) Wiley-Liss, New York, pp. 55-76).

Phytic acid is degraded in plant cells to D-myo-inositol 1,2,4,5,6-pentakisphosphate and orthophosphate through the action of phytase. Manipulation of this enzyme activity could lead to a reduction of phytic acid levels in seeds and an increase in inositol trisphosphate and free phosphate, thus making phosphorus more metabolically available to

animals that are fed the seed. Another method to lower phytic acid levels is by inhibiting the activity of myo-inositol-1(or 4)-monophosphatase, which catalyzes the reaction: myo-inositol 1-phosphate + H2O = myo-inositol + orthophosphate. Manipulation of the activity of this enzyme in developing seeds could decrease phytic acid levels in seeds and increase levels of free phosphate. Lastly, phytic acid levels could also be reduced by inhibiting the activity of inositol trisphosphate kinase. This enzyme catalyzes the reaction: ATP + 1D-myo-inositol 1,3,4-trisphosphate = ADP + 1D-myo-inositol 1,3,4,6-tetrakisphosphate. This reaction is one of the final steps leading to the formation of Myo-Inositol 1,2,3,4,5,6-hexaphosphate (phytic acid). Reduction in the activity of the enzyme in developing seeds would interrupt phytic acid synthesis leaving the phosphate as the more metabolically available inositol trisphosphate and free phosphate.

In the United States, corn accounts for about 80% of the grain fed to all classes of livestock, including poultry, and is usually ground before feeding (Corn: Chemistry and Technology, 1987, American Association of Cereal Chemists, Inc., Edited by Stanley A. Watson and Paul E. Ramstad). A meal with decreased amounts of phytic acid and increased amounts of available phosphate would lead to improved feed efficiency in corn-containing rations, making available certain minerals especially zinc, magnesium, iron and calcium. Indeed, enzymatic treatment of soybean meal-containing rations to partially hydrolyze the phosphate groups from phytic acid improves both phosphate availability and the availability of other limiting nutrients. Also, in the wet milling of corn, phytate in the steepwater tends to precipitate, causing problems in handling, storing and transportation of the steep liquor. (Pen et al. (1993) *Biotechnology 11:*811-814). In light of these factors, it is apparent that corn plants with heritable, substantially reduced levels of phytic acid and increased levels of free phosphorous in their seeds would be desirable. Accordingly, the availability of nucleic acid sequences encoding all or a portion of these enzymes would facilitate studies to better understand carbohydrate metabolism and function in plants, provide genetic tools for the manipulation of these biosynthetic pathways, and provide a means to control carbohydrate transport and distribution in plant cells.

SUMMARY OF THE INVENTION

The instant invention relates to isolated nucleic acid fragments encoding phytic acid biosynthetic enzymes. Specifically, this invention concerns an isolated nucleic acid fragment encoding a myo-inositol-1 (or 4)-monophosphatase or a plant homolog of the *Synechocystis sp.* extragenic suppressor protein, a protein in the inositol monophosphatase family of proteins (Keneko, T., et al., (1996) *DNA Res. 3(3)*:109-136). In addition, this invention relates to a nucleic acid fragment that is complementary to the nucleic acid fragment encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein.

An additional embodiment of the instant invention pertains to a polypeptide encoding all or a substantial portion of a phytic acid biosynthetic enzyme selected from the group consisting of myo-inositol-1 (or 4)-monophosphatase and extragenic suppressor proteins.

In another embodiment, the instant invention relates to a chimeric gene encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein, or to a chimeric gene that comprises a nucleic acid fragment that is complementary to a nucleic acid fragment encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein, operably linked to suitable regulatory sequences, wherein expression of the chimeric gene results in production of levels of the encoded protein in a transformed host cell that is altered (i.e., increased or decreased) from the level produced in an untransformed host cell.

In a further embodiment, the instant invention concerns a transformed host cell comprising in its genome a chimeric gene encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein, operably linked to suitable regulatory sequences. Expression of the chimeric gene results in production of altered levels of the encoded protein in the transformed host cell. The transformed host cell can be of eukaryotic or prokaryotic origin, and include cells derived from higher plants and microorganisms. The invention also includes transformed plants that arise from transformed host cells of higher plants, and seeds derived from such transformed plants.

An additional embodiment of the instant invention concerns a method of altering the level of expression of a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein in a transformed host cell comprising: a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein; and b) growing the transformed host cell under conditions that are suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of altered levels of myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein in the transformed host cell.

An addition embodiment of the instant invention concerns a method for obtaining a nucleic acid fragment encoding all or a substantial portion of an amino acid sequence encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing which form a part of this application.

Figure 1 presents an alignment of the amino acid sequence set forth in SEQ ID NOs:2, 4, 6 and 8 with the *Lycopersicon esculentum* IMP amino acid sequences (SEQ ID NO:21 and 22). Alignments were performed using the Clustal algorithm.

Figure 2 presents an alignment of the amino acid sequence set forth in SEQ ID NOs:10, 12, 14, 16, 18 and 20 with the *Synechocystis sp.* extragenic suppressor protein

amino acid sequences (SEQ ID NO:23 and 24). Alignments were performed using the Clustal algorithm.

The following sequence descriptions and Sequence Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

SEQ ID NO:1 is the nucleotide sequence comprising a portion of the cDNA insert in clone rl0n.pk127.f22 encoding a portion of a rice myo-inositol-1 (or 4)-monophosphatase.

SEQ ID NO:2 is the deduced amino acid sequence of a portion of a myo-inositol-1 (or 4)-monophosphatase derived from the nucleotide sequence of SEQ ID NO:1.

SEQ ID NO:3 is the nucleotide sequence comprising a portion of the cDNA insert in clone sfl1.pk0034.a12(5') encoding a portion of a soybean myo-inositol-1 (or 4)-monophosphatase.

SEQ ID NO:4 is the deduced amino acid sequence of a portion of a myo-inositol-1 (or 4)-monophosphatase derived from the nucleotide sequence of SEQ ID NO:3.

SEQ ID NO:5 is the nucleotide sequence comprising a portion of the cDNA insert in clone sfl1.pk0034.a12(3') encoding a portion of a soybean myo-inositol-1 (or 4)-monophosphatase.

SEQ ID NO:6 is the deduced amino acid sequence of a portion of a myo-inositol-1 (or 4)-monophosphatase derived from the nucleotide sequence of SEQ ID NO:5.

SEQ ID NO:7 is the nucleotide sequence comprising a the entire cDNA insert in clone wlmk1.pk0020.a9 encoding a wheat myo-inositol-1 (or 4)-monophosphatase.

SEQ ID NO:8 is the deduced amino acid sequence of a myo-inositol-1 (or 4)-monophosphatase derived from the nucleotide sequence of SEQ ID NO:7.

SEQ ID NO:9 is the nucleotide sequence comprising a portion of the cDNA insert in clone bsh1.pk0007.g11 encoding a portion of a barley extragenic suppressor protein.

SEQ ID NO:10 is the deduced amino acid sequence of a portion of an extragenic suppressor protein derived from the nucleotide sequence of SEQ ID NO:9.

SEQ ID NO:11 is the nucleotide sequence comprising a portion of the cDNA insert in clone cco1n.pk066.p15 encoding a portion of a corn extragenic suppressor protein.

SEQ ID NO:12 is the deduced amino acid sequence of a portion of an extragenic suppressor protein derived from the nucleotide sequence of SEQ ID NO:11.

SEQ ID NO:13 is the nucleotide sequence comprising the entire cDNA insert in clone cdt2c.pk003.b20 encoding a corn extragenic suppressor protein.

SEQ ID NO:14 is the deduced amino acid sequence of a portion of an extragenic suppressor protein derived from the nucleotide sequence of SEQ ID NO:13.

SEQ ID NO:15 is the nucleotide sequence comprising a portion of the cDNA insert in clone rl0n.pk0062.c6 encoding a portion of a rice extragenic suppressor protein.

SEQ ID NO:16 is the deduced amino acid sequence of a portion of an extragenic suppressor protein derived from the nucleotide sequence of SEQ ID NO:15.

SEQ ID NO:17 is the nucleotide sequence comprising a contig assembled from portions of the cDNA inserts in clones sl2.pk122.p24, src3c.pk013.g15 and sfl1n.pk003.g19 encoding a soybean extragenic suppressor protein.

SEQ ID NO:18 is the deduced amino acid sequence of an extragenic suppressor protein derived from the nucleotide sequence of SEQ ID NO:17.

SEQ ID NO:19 is the nucleotide sequence comprising a portion of the cDNA insert in clone wlm0.pk0010.f6 encoding a portion of a wheat extragenic suppressor protein.

SEQ ID NO:20 is the deduced amino acid sequence of a portion of an extragenic suppressor protein derived from the nucleotide sequence of SEQ ID NO:19.

SEQ ID NO:21 is the amino acid sequence of myo-inositol-1 (or 4)-monophosphatase from *Lycopersicon esculentum* (NCBI Identification No. gi 1709203).

SEQ ID NO:22 is the amino acid sequence of myo-inositol-1 (or 4)-monophosphatase from *Lycopersicon esculentum* (NCBI Identification No. gi 1709205).

SEQ ID NO:23 is the amino acid sequence of extragenic suppressor protein from *Synechocystis sp.* (NCBI Identification No. gi 3915048).

SEQ ID NO:24 is the amino acid sequence of extragenic suppressor protein from *Synechocystis sp.* (NCBI Identification No. gi 1652942).

The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUBMB standards described in *Nucleic Acids Research 13*:3021-3030 (1985) and in the *Biochemical Journal 219 (No. 2)*:345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

DETAILED DESCRIPTION OF THE INVENTION

In the context of this disclosure, a number of terms shall be utilized. As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA. As used herein, "contig" refers to an assemblage of overlapping nucleic acid sequences to form one contiguous nucleotide sequence. For example, several DNA sequences can be compared and aligned to identify common or overlapping regions. The individual sequences can then be assembled into a single contiguous nucleotide sequence.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotide bases results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence.

"Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotide bases does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially

similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate alteration of gene expression by antisense or co-suppression technology or alteration of the functional properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less than the entire coding region of a gene, and by nucleic acid fragments that do not share 100% sequence identity with the gene to be suppressed. Moreover, alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded protein, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products.

Moreover, substantially similar nucleic acid fragments may also be characterized by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the nucleic acid fragments disclosed herein.

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent similarity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Preferred are those nucleic acid fragments whose nucleotide sequences encode amino acid sequences that are 80% similar to the amino acid sequences reported herein. More preferred nucleic acid fragments encode amino acid sequences that are 90% similar to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are 95% similar to the amino acid sequences reported herein. Sequence alignments and percent similarity calculations were performed using the Megalign program of the LASARGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins, D. G. and Sharp, P. M. (1989) *CABIOS.* *5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10), (hereafter Clustal algorithm). Default parameters for pairwise alignments

using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol. 215*:403-410; see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches partial or complete amino acid and nucleotide sequences encoding one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins as set forth in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18 and 20. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as related to a sequence

of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as

"constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg, (1989) *Biochemistry of Plants 15*:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a DNA sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D. (1995) *Molecular Biotechnology 3*:225).

The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) *Plant Cell 1*:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Patent No. 5,107,065, incorporated herein by reference). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to sense RNA, antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020, incorporated herein by reference).

"Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels, J. J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys. 100*:1627-1632).

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include Agrobacterium-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol. 143*:277) and particle-accelerated or "gene gun" transformation technology (Klein et al. (1987) *Nature (London) 327*:70-73; U.S. Patent No. 4,945,050, incorporated herein by reference).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Maniatis").

Nucleic acid fragments encoding at least a portion of several phytic acid biosynthetic enzymes have been isolated and identified by comparison of random plant cDNA sequences to public databases containing nucleotide and protein sequences using the BLAST algorithms well known to those skilled in the art. Table 1 lists the proteins that are described herein, and the designation of the cDNA clones that comprise the nucleic acid fragments encoding these proteins.

TABLE 1

Phytic Acid Biosynthetic Enzymes

Enzyme	Clone	Plant
Myo-inositol-1 (or 4)-monophosphatase 1	rl0n.pk127.f22	Rice
	sfl1.pk0034.a12(5')	Soybean
	sfl1.pk0034.a12(3")	Soybean
	wlmk1.pk0020.a9	wheat
Extragenic suppressor protein	bsh1.pk0007.g11	Barley
	cco1n.pk066.p15	Corn
	cdt2c.pk003.b20	Corn
	rl0n.pk0062.c6	Rice
	sl2.pk122.p24	Soybean
	src3c.pk013.g15	Soybean
	sfl1n.pk003.g19	Soybean
	wlm0.pk0010.f6	Wheat

The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous proteins from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding other myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labeling, nick translation, or end-labeling techniques, or RNA probes using available *in vitro* transcription systems. In

addition, specific primers can be designed and used to amplify a part or all of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., (1988) *PNAS USA 85*:8998) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., (1989) *PNAS USA 86*:5673; Loh et al., (1989) *Science 243*:217). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman, M. A. and Martin, G. R., (1989) *Techniques 1*:165).

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner, R. A. (1984) *Adv. Immunol. 36*:1; Maniatis).

The nucleic acid fragments of the instant invention may be used to create transgenic plants in which the disclosed myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins are present at higher or lower levels than normal or in cell types or developmental stages in which they are not normally found. This would have the effect of altering the level of phytic acid biosynthesis in those cells.

Overexpression of the myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins of the instant invention may be accomplished by first constructing a chimeric gene in which the coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of development. For reasons of convenience, the chimeric gene may comprise promoter sequences and translation leader sequences derived from the same genes. 3' Non-coding sequences encoding

transcription termination signals may also be provided. The instant chimeric gene may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant chimeric gene can then constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J. 4*:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics 218*:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant phytic acid biosynthetic enzymes to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that the chimeric gene described above may be further supplemented by altering the coding sequence to encode myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein with appropriate intracellular targeting sequences such as transit sequences (Keegstra, K. (1989) *Cell 56*:247-253), signal sequences or sequences encoding endoplasmic reticulum localization (Chrispeels, J. J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53), or nuclear localization signals (Raikhel, N. (1992) *Plant Phys. 100*:1627-1632) added and/or with targeting sequences that are already present removed. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of utility may be discovered in the future.

It may also be desirable to reduce or eliminate expression of genes encoding myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein in plants for some applications. In order to accomplish this, a chimeric gene designed for co-suppression of the instant phytic acid biosynthetic enzymes can be constructed by linking a gene or gene fragment encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein to plant promoter sequences. Alternatively, a chimeric gene designed to express antisense RNA for all or part of the instant nucleic acid fragment can be constructed by linking the gene or gene fragment in reverse orientation to plant promoter sequences. Either the co-suppression or antisense chimeric genes could be introduced into plants via transformation wherein expression of the corresponding endogenous genes are reduced or eliminated.

The instant myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins (or portions thereof) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to the these proteins by methods well known to those skilled in the art. The antibodies are useful for detecting myo-inositol-1 (or

4)-monophosphatase or extragenic suppressor proteins *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct a chimeric gene for production of the instant myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins. This chimeric gene could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded phytic acid biosynthetic enzymes. An example of a vector for high level expression of the instant myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor proteins in a bacterial host is provided (Example 7).

All or a substantial portion of the nucleic acid fragments of the instant invention may also be used as probes for genetically and physically mapping the genes that they are a part of, and as markers for traits linked to those genes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes. For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with the nucleic acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et at., (1987) *Genomics 1*:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein, D. et al., (1980) *Am. J. Hum. Genet. 32*:314-331).

The production and use of plant gene-derived probes for use in genetic mapping is described in R. Bernatzky, R. and Tanksley, S. D. (1986) *Plant Mol. Biol. Reporter 4(1)*:37-41. Numerous publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2 intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to those skilled in the art.

Nucleic acid probes derived from the instant nucleic acid sequences may also be used for physical mapping (i.e., placement of sequences on physical maps; *see* Hoheisel, J. D., et al., In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

In another embodiment, nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping (Trask,

B. J. (1991) *Trends Genet. 7*:149-154). Although current methods of FISH mapping favor use of large clones (several to several hundred KB; see Laan, M. et al. (1995) *Genome Research 5*:13-20), improvements in sensitivity may allow performance of FISH mapping using shorter probes.

A variety of nucleic acid amplification-based methods of genetic and physical mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification (Kazazian, H. H. (1989) *J. Lab. Clin. Med. 114(2)*:95-96), polymorphism of PCR-amplified fragments (CAPS; Sheffield, V. C. et al. (1993) *Genomics 16*:325-332), allele-specific ligation (Landegren, U. et al. (1988) *Science 241*:1077-1080), nucleotide extension reactions (Sokolov, B. P. (1990) *Nucleic Acid Res. 18*:3671), Radiation Hybrid Mapping (Walter, M. A. et al. (1997) *Nature Genetics 7*:22-28) and Happy Mapping (Dear, P. H. and Cook, P. R. (1989) *Nucleic Acid Res. 17*:6795-6807). For these methods, the sequence of a nucleic acid fragment is used to design and produce primer pairs for use in the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the instant nucleic acid sequence. This, however, is generally not necessary for mapping methods.

Loss of function mutant phenotypes may be identified for the instant cDNA clones either by targeted gene disruption protocols or by identifying specific mutants for these genes contained in a maize population carrying mutations in all possible genes (Ballinger and Benzer, (1989) *Proc. Natl. Acad. Sci USA 86*:9402; Koes et al., (1995) *Proc. Natl. Acad. Sci USA 92*:8149; Bensen et al., (1995) *Plant Cell 7*:75). The latter approach may be accomplished in two ways. First, short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols in conjunction with a mutation tag sequence primer on DNAs prepared from a population of plants in which Mutator transposons or some other mutation-causing DNA element has been introduced (see Bensen, *supra*). The amplification of a specific DNA fragment with these primers indicates the insertion of the mutation tag element in or near the plant gene encoding the myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein. Alternatively, the instant nucleic acid fragment may be used as a hybridization probe against PCR amplification products generated from the mutation population using the mutation tag sequence primer in conjunction with an arbitrary genomic site primer, such as that for a restriction enzyme site-anchored synthetic adaptor. With either method, a plant containing a mutation in the endogenous gene encoding a myo-inositol-1 (or 4)-monophosphatase or extragenic suppressor protein can be identified and obtained. This mutant plant can then be used to determine or confirm the natural function of the gene product.

EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of cDNA Libraries; Isolation and Sequencing of cDNA Clones

cDNA libraries representing mRNAs from various barley, corn, rice, soybean and wheat tissues were prepared. The characteristics of the libraries are described below.

TABLE 2

cDNA Libraries from Barley, Corn, Rice, Soybean and Wheat

Library	Tissue	Clone
bsh1	Barley sheath, developing seedling	bsh1.pk0007.g11
cco1n	Corn (Zea mays L.) cob of 67 day old plants grown in green house*	cco1n.pk066.p15(3')
cdt2c	Corn (Zea mays L.) developing tassel	cdt2c.pk003.b20
rl0n	Rice (Oryza sativa L.) 15 day leaf*	rl0n.pk0062.c6
		rl0n.pk127.f22
sfl1	Soybean (Glycine max L.) immature flower	sfl1.pk0034.a12(5')
		sfl1.pk0034.a12(3")
sfl1n	Soybean (Glycine max L.) immature flower*	sfl1n.pk003.g19
sl2	Soybean (Glycine max L.) two week old developing seedlings treated with 2.5 ppm chlorimuron	sl2.pk122.p24
src3c	Soybean (Glycine max L., Bell) 8 day old root inoculated with eggs of cyst nematode Heterodera glycines (Race 14) for 4 days.	src3c.pk013.g15
wlm0	Wheat (Triticum aestivum L.) seedlings 0 hr after inoculation with Erysiphe graminis f. sp tritici	wlm0.pk0010.f6
wlmk1	Wheat (Triticum aestivum L.) seedlings 1 hr after inoculation with Erysiphe graminis f. sp tritici and treatment with fungicide**	wlmk1.pk0020.a9

*These libraries were normalized essentially as described in U.S. Patent No. 5,482,845

**Application of 6-iodo-2-propoxy-3-propyl-4(3*H*)-quinazolinone; synthesis and methods of using this compound are described in USSN 08/545,827, incorporated herein by reference.

cDNA libraries were prepared in Uni-ZAP™ XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). Conversion of the

Uni-ZAP™ XR libraries into plasmid libraries was accomplished according to the protocol provided by Stratagene. Upon conversion, cDNA inserts were contained in the plasmid vector pBluescript. cDNA inserts from randomly picked bacterial colonies containing recombinant pBluescript plasmids were amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences or plasmid DNA was prepared from cultured bacterial cells. Amplified insert DNAs or plasmid DNAs were sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams, M. D. et al., (1991) *Science 252*:1651). The resulting ESTs were analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 2

Identification of cDNA Clones

ESTs encoding phytic acid biosynthetic enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol. 215*:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. (1993) *Nature Genetics 3*:266-272 and Altschul, Stephen F., et al. (1997) *Nucleic Acids Res. 25*:3389-3402) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

EXAMPLE 3

Characterization of cDNA Clones Encoding Myo-Inositol-1 (or 4)-Monophosphatase Homologs

The BLASTX search using the EST sequences from clones rl0n.pk127.f22 and sfl1.pk0034.a12(3') revealed similarity of the proteins encoded by the cDNAs to myo-inositol-1 (or 4)-monophosphatase 1 from *Lycopersicon esculentum*. (NCBI Identification No. gi 1709203). The BLASTX search using the EST sequences from clones sfl1.pk0034.a12(5') and wlmk1.pk0020.a9 revealed similarity of the proteins encoded by the cDNAs to myo-inositol-1 (or 4)-monophosphatase 3 from *Lycopersicon esculentum*. (NCBI Identification No. gi 1709205).

The BLAST results for each of these ESTs are shown in Table 3:

TABLE 3

BLAST Results for Clones Encoding Polypeptides Homologous to *Lycopersicon esculentum* Myo-Inositol-1 (or 4)-Monophosphatase Proteins

Clone	BLAST pLog Score
rl0n.pk127.f22	54.40
sfl1.pk0034.a12(5')	89.00
sfl1.pk0034.a12(3')	23.70
wlmk1.pk0020.a9	130.00

The sequence of a portion of the cDNA insert from clone rl0n.pk127.f22 is shown in SEQ ID NO:1; the deduced amino acid sequence of this cDNA, which represents 42% of the of the protein (N-terminal region), is shown in SEQ ID NO:2. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:2 and the *Lycopersicon esculentum* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:2 is 77% similar to the *Lycopersicon esculentum* IMP-1 protein.

The sequence of a portion of the cDNA insert from clone sfl1.pk0034.a12(5') is shown in SEQ ID NO:3; the deduced amino acid sequence of this cDNA, which represents 63% of the of the protein (N-terminal region), is shown in SEQ ID NO:4. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:4 and the *Lycopersicon esculentum* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:4 is 74% similar to the *Lycopersicon esculentum* IMP-3 protein.

The sequence of a portion of the cDNA insert from clone sfl1.pk0034.a12(3') is shown in SEQ ID NO:5; the deduced amino acid sequence of this cDNA, which represents 27% of the of the protein (C-terminal region), is shown in SEQ ID NO:6. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:6 and the *Lycopersicon esculentum* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:6 is 58% similar to the *Lycopersicon esculentum* IMP-1 protein.

The sequence of the entire cDNA insert from clone wlmk1.pk0020.a9 is shown in SEQ ID NO:7; the deduced amino acid sequence of this cDNA, which represents 100% of the of the protein, is shown in SEQ ID NO:8. The amino acid sequence set forth in SEQ ID NO:8 was evaluated by BLASTP, yielding a pLog value of 113.00 versus the *Lycopersicon esculentum* sequence. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:8 and the *Lycopersicon esculentum* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO: is 69% similar to the *Lycopersicon esculentum* IMP-3 protein.

Figure 1 presents an alignment of the amino acid sequence set forth in SEQ ID NOs:2, 4, 6 and 8 with the *Lycopersicon esculentum* IMP amino acid sequences, SEQ ID NO:21 and 22. Alignments were performed using the Clustal algorithm.

These sequences represent the first rice, soybean and wheat sequences encoding myo-inositol-1 (or 4)-monophosphatase proteins.

EXAMPLE 4

Characterization of cDNA Clones Encoding Extragenic Suppressor Proteins

The BLASTX search using the EST sequences from clones bsh1.pk0007.g11, cco1n.pk066.p15 and rl0n.pk0062.c6 revealed similarity of the proteins encoded by the cDNAs to extragenic suppressor protein from *Synechocystis sp.* (NCBI Identification No. gi 3915048). The BLASTX search using the EST sequences from clones cdt2c.pk003.b20, sl2.pk122.p24, src3c.pk013.g15, sfl1n.pk003.g19 and wlm0.pk0010.f6 revealed similarity of the proteins encoded by the cDNAs to extragenic suppressor protein from *Synechocystis sp.* (NCBI Identification No. gi 1652942).

In the process of comparing the ESTs it was found that soybean clones sl2.pk122.p24, src3c.pk013.g15 and sfl1n.pk003.g19 had overlapping regions of homology. Using this homology it was possible to align the ESTs and assemble a contig encoding a unique soybean extragenic suppressor protein.

The BLAST results for each of the ESTs and the soybean contig are shown in Table 4:

TABLE 4

BLAST Results for Clones Encoding Polypeptides Homologous to *Synechocystis sp* Extragenic Suppressor Protein

Clone	BLAST pLog Score
bsh1.pk0007.g11	46.10
cco1n.pk066.p15	21.70
cdt2c.pk003.b20	30.40
rl0n.pk0062.c6	22.30
Contig composed of clones: sl2.pk122.p24 src3c.pk013.g15 sfl1n.pk003.g19 wlm0.pk0010.f6	24.70 29.40

The sequence of a portion of the cDNA insert from clone bsh1.pk0007.g11 is shown in SEQ ID NO:9; the deduced amino acid sequence of this cDNA, which represents 74% of the of the protein (C-terminal region), is shown in SEQ ID NO:10. The amino acid sequence set forth in SEQ ID NO:10 was evaluated by BLASTP, yielding a pLog value of 40.30 versus the *Synechocystis sp.* sequence. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:10 and the *Synechocystis sp.* sequence (using the Clustal

algorithm) revealed that the protein encoded by SEQ ID NO:10 is 34% similar to the *Synechocystis sp.* extragenic suppressor protein.

The sequence of a portion of the cDNA insert from clone cco1n.pk066.p15 is shown in SEQ ID NO:11; the deduced amino acid sequence of this cDNA, which represents 40% of the of the protein (C-terminal region), is shown in SEQ ID NO:12. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:12 and the *Synechocystis sp.* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:12 is 34% similar to the *Synechocystis sp.* extragenic suppressor protein.

The sequence of the entire cDNA insert from clone cdt2c.pk003.b20 is shown in SEQ ID NO:13; the deduced amino acid sequence of this cDNA, which represents 100% of the of the protein, is shown in SEQ ID NO:14. The amino acid sequence set forth in SEQ ID NO:14 was evaluated by BLASTP, yielding a pLog value of 34.70 versus the *Synechocystis sp.* sequence. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:14 and the *Synechocystis sp.* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:14 is 28% similar to the *Synechocystis sp.* extragenic suppressor protein.

The sequence of a portion of the cDNA insert from clone rl0n.pk0062.c6 is shown in SEQ ID NO:15; the deduced amino acid sequence of this cDNA, which represents 42% of the of the protein (C-terminal region), is shown in SEQ ID NO:16. The amino acid sequence set forth in SEQ ID NO:16 was evaluated by BLASTP, yielding a pLog value of 18.52 versus the *Synechocystis sp.* sequence. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:16 and the *Synechocystis sp.* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:16 is 33% similar to the *Synechocystis sp.* extragenic suppressor protein.

The sequence of the soybean contig composed of clones sl2.pk122.p24, src3c.pk013.g15 and sfl1n.pk003.g19 is shown in SEQ ID NO:17; the deduced amino acid sequence of this cDNA, which represents 100% of the of the protein, is shown in SEQ ID NO:18. The amino acid sequence set forth in SEQ ID NO:18 was evaluated by BLASTP, yielding a pLog value of 32.00 versus the *Synechocystis sp.* sequence. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:18 and the *Synechocystis sp.* sequence (using the Clustal algorithm) revealed that the protein encoded by SEQ ID NO:18 is 26% similar to the *Synechocystis sp.* extragenic suppressor protein.

The sequence of a portion of the cDNA insert from clone wlm0.pk0010.f6 is shown in SEQ ID NO:19; the deduced amino acid sequence of this cDNA, which represents 96% of the of the protein, is shown in SEQ ID NO:20. The amino acid sequence set forth in SEQ ID NO:20 was evaluated by BLASTP, yielding a pLog value of 35.22 versus the *Synechocystis sp.* sequence. A calculation of the percent similarity of the amino acid sequence set forth in SEQ ID NO:20 and the *Synechocystis sp.* sequence (using the Clustal algorithm) revealed

that the protein encoded by SEQ ID NO:20 is 25% similar to the *Synechocystis sp.* extragenic suppressor protein.

Figure 2 presents an alignment of the amino acid sequence set forth in SEQ ID NOs:10, 12, 14, 16, 18 and 20 with the *Synechocystis sp.* extragenic suppressor protein amino acid sequences, SEQ ID NO:23 and 24. Alignments were performed using the Clustal algorithm.

These sequences represent the first plant sequences encoding extragenic suppressor proteins.

EXAMPLE 5

Expression of Chimeric Genes in Monocot Cells

A chimeric gene comprising a cDNA encoding phytic acid biosynthetic enzyme in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (NcoI or SmaI) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes NcoI and SmaI and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli* XL1-Blue (Epicurian Coli XL-1 Blue™; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (Sequenase™ DNA Sequencing Kit; U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding a phytic acid biosynthetic enzyme, and the 10 kD zein 3' region.

The chimeric gene described above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al., (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable

embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al., (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 μm in diameter) are coated with DNA using the following technique. Ten μg of plasmid DNAs are added to 50 μL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 μL of a 2.5 M solution) and spermidine free base (20 μL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 μL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 μL of ethanol. An aliquot (5 μL) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains gluphosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the glufosinate-

supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., (1990) *Bio/Technology 8*:833-839).

EXAMPLE 6

Expression of Chimeric Genes in Dicot Cells

A seed-specific expression cassette composed of the promoter and transcription terminator from the gene encoding the β subunit of the seed storage protein phaseolin from the bean *Phaseolus vulgaris* (Doyle et al. (1986) *J. Biol. Chem. 261*:9228-9238) can be used for expression of the instant phytic acid biosynthetic enzymes in transformed soybean. The phaseolin cassette includes about 500 nucleotides upstream (5') from the translation initiation codon and about 1650 nucleotides downstream (3') from the translation stop codon of phaseolin. Between the 5' and 3' regions are the unique restriction endonuclease sites Nco I (which includes the ATG translation initiation codon), Sma I, Kpn I and Xba I. The entire cassette is flanked by Hind III sites.

The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the expression vector. Amplification is then performed as described above, and the isolated fragment is inserted into a pUC18 vector carrying the seed expression cassette.

Soybean embroys may then be transformed with the expression vector comprising a sequence encoding the phytic acid biosynthetic enzyme. To induce somatic embryos, cotyledons, 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, can be cultured in the light or dark at 26°C on an appropriate agar medium for 6-10 weeks. Somatic embryos which produce secondary embryos are then excised and placed into a suitable liquid medium. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions are maintained as described below.

Soybean embryogenic suspension cultures can maintained in 35 mL liquid media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures are subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of liquid medium.

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Kline et al. (1987) *Nature* (London) *327*:70, U.S. Patent No. 4,945,050). A DuPont Biolistic™ PDS1000/HE instrument (helium retrofit) can be used for these transformations.

A selectable marker gene which can be used to facilitate soybean transformation is a chimeric gene composed of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature 313*:810-812), the hygromycin phosphotransferase gene from plasmid pJR225 (from *E. coli*; Gritz et al.(1983) *Gene 25*:179-188) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. The seed expression cassette comprising the phaseolin 5' region, the fragment encoding the phytic acid biosynthetic enzyme and the phaseolin 3' region can be isolated as a restriction fragment. This fragment can then be inserted into a unique restriction site of the vector carrying the marker gene.

To 50 μL of a 60 mg/mL 1 μm gold particle suspension is added (in order): 5 μL DNA (1 μg/μL), 20 μl spermidine (0.1 M), and 50 μL $CaCl_2$ (2.5 M). The particle preparation is then agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles are then washed once in 400 μL 70% ethanol and resuspended in 40 μL of anhydrous ethanol. The DNA/particle suspension can be sonicated three times for one second each. Five μL of the DNA-coated gold particles are then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture is placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue are normally bombarded. Membrane rupture pressure is set at 1100 psi and the chamber is evacuated to a vacuum of 28 inches mercury. The tissue is placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue can be divided in half and placed back into liquid and cultured as described above.

Five to seven days post bombardment, the liquid media may be exchanged with fresh media, and eleven to twelve days post bombardment with fresh media containing 50 mg/mL hygromycin. This selective media can be refreshed weekly. Seven to eight weeks post bombardment, green, transformed tissue may be observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue is removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Each new line may be treated as an independent transformation event. These suspensions can then be subcultured and maintained as clusters of immature embryos or regenerated into whole plants by maturation and germination of individual somatic embryos.

EXAMPLE 7

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant phytic acid biosynthetic enzymes can be inserted into the T7 *E. coli* expression vector pBT430. This vector is a derivative of pET-3a (Rosenberg et al. (1987) *Gene 56*:125-135) which employs the bacteriophage T7 RNA polymerase/T7 promoter system. Plasmid pBT430 was constructed by first destroying the EcoR I and Hind III sites in pET-3a at their original positions. An oligonucleotide adaptor containing

EcoR I and Hind III sites was inserted at the BamH I site of pET-3a. This created pET-3aM with additional unique cloning sites for insertion of genes into the expression vector. Then, the Nde I site at the position of translation initiation was converted to an Nco I site using oligonucleotide-directed mutagenesis. The DNA sequence of pET-3aM in this region, 5'-CATATGG, was converted to 5'-CCCATGG in pBT430.

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 μg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 μL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as decribed above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 μg/mL ampicillin. Transformants containing the gene encoding the phytic acid biosynthetic enzyme are then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

For high level expression, a plasmid clone with the cDNA insert in the correct orientation relative to the T7 promoter can be transformed into *E. coli* strain BL21(DE3) (Studier et al. (1986) *J. Mol. Biol. 189*:113-130). Cultures are grown in LB medium containing ampicillin (100 mg/L) at 25°C. At an optical density at 600 nm of approximately 1, IPTG (isopropylthio-β-galactoside, the inducer) can be added to a final concentration of 0.4 mM and incubation can be continued for 3 h at 25°. Cells are then harvested by centrifugation and re-suspended in 50 μL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One μg of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.